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FINAL REPORT

Study Title

In Vivo Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Test Article

Ethylenediamine Dinitrate (EDDN)

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Laboratory Project I.D.

SITEK Study No. 1003-1521

Study Initiation Date

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Sponsor

USA RDECOM, AMSRD-MSF
Environmental Acquisition & Logistics Sustaining Program
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Ethylenediamine dinitrate (EDDN) (99.5% pure) was tested forclastogenic potentials to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and femal CD-1 mice according to OECD TG 474 in compliance with Good Laboratory Practice. Male and female mice were dosed orally at 500, 1000, and 2000 mg/kg. Animals were euthanized approximately 24 and 48 hr after dosing. The percentage of polychromatic erythrocytes (PCE) and frequency of micronucleated polychromatic erythrocyte (MPCE) were determined at 24 and 48 hours. Cytotoxicity was assessed by scoring the number of PCE and norchromatic erythrocyte (NCE) in first 200 erythrocytes for each animal. There were not statistically significant increases in the number of MPCE in the treated groups when compared to the concurrent vehilce control group. These results indicate that EDDN was negative in the in vivo mouse micronucleus assay and therefore is not considered to be a clastogenic agent.

15. SUBJECT TERMS

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STUDY DIRECTOR'S COMPLIANCE STATEMENT

Study No.: 1003-1521
Sponsor's Test Article I.D.: Ethylenediamine Dinitrate (EDDN)

The study described in this report was conducted in compliance with the following test guidelines:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Section 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002, (1).

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997, (2).

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996, (3).

The study described in this report was conducted in compliance with the following Good Laboratory Practice standard, except as indicated in the last paragraph:

United States Food and Drug Administration, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005, (4).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005, (5).

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 Nohsan, Notification No. 6283, October 1, 1999, (6).

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997, (7).

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984 (8).

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998, (9).

The strength and stability of the test article, dosing solutions and controls, under the experimental conditions, were not determined.

Signature _____ 2-25-10

Paul E. Kirby, Ph.D.[1] Date

Study Director

[1] Dr. Jian Song was the Study Director for the in-life phase of this study and was the author of the draft report. He was not in the employ of SITEK Research Laboratories when this final report was prepared, therefore, Dr. Kirby has replaced him as Study Director.

QUALITY ASSURANCE UNIT'S STATEMENT

Study No.: 1003-1521
Sponsor's Test Article I.D.: Ethylenediamine Dinitrate (EDDN)

The performance of this study was audited for adherence to the Good Laboratory Practice regulations for nonclinical laboratory studies by the Quality Assurance Unit of SITEK Research Laboratories. In this context, the facilities, equipment, personnel, methods, practices, controls, original data and reports have been inspected as per SITEK's Quality Assurance Unit's Standard Operating Procedures. The information contained within this report accurately reflects the raw data generated from this study.

Protocol Review Date: 08/05/09

The following phases were inspected for this study:

Inspection Date	Phases Inspected	Date Findings Reported to Study Director	Date Findings Reported to Management
08/21/09	Randomization	08/21/0	08/21/09
09/29/09	Workbook Audit	09/29/09	10/02/09
10/01/09	Draft Report	10/02/09	10/02/09
02/25/10	Final Report	02/25/10	02/25/10

Signature: _____ Date: 2/25/10

Ray Kirby, B.S.
Quality Assurance

STUDY DIRECTOR'S SIGNATURE PAGE

This study was performed under the supervision of Jian Song, Ph.D., Study Director for In Vivo Micronucleus Studies, at SITEK Research Laboratories, 15235 Shady Grove Road, Suite 303, Rockville, Maryland 20850.

The Draft Report on this study was written by Karen S.K. Shore, B.A., and released by the Study Director, Jian Song, on October 2, 2009. The Final Report was prepared by Dr. Paul E. Kirby[2] and released on February 25, 2010.

Signature

Paul E. Kirby, Ph.D.
Study Director

2-25-10
Date

[2] Dr. Song was no longer in the employ of SITEK Research Laboratories when the final report was prepared, therefore, Dr. Kirby has replaced him as Study Director.

ABSTRACT

The test article, Ethylenediamine Dinitrate (EDDN, 99.5% pure), was tested for its potential to induce micronucleated polychromatic erythrocytes (MPCE) in the bone marrow cells of male and female CD-1 mice. Deionized distilled water was used as the vehicle control. The test article and vehicle control were administered by oral gavage. Three male and three female mice per dose group were used in the Range Finding Test. Five male and five female mice per dose group per harvest time were used in the Micronucleus Assay.

The Range Finding Test was performed at 10, 50, 100, 500, 1000 and 2000 mg/kg. No abnormal clinical signs or weight loss were observed in any animals during the three day observation period.

Test article doses of 500, 1000 and 2000 mg/kg body weight for the males and females in the Micronucleus Assay were selected based on the results of the Range Finding Test. The highest dose was selected to exclude lethality in accordance with U.S. E.P.A., OECD and ICH guidance documents. The positive control group received a single, oral gavage dose of cyclophosphamide (CP) at 80 mg/kg body weight. Animals were euthanized approximately 24 and 48 hours after dose administration. Positive controls were included in the 24-hour harvest only.

In the Micronucleus Assay, the percentage of polychromatic erythrocytes (PCE) and frequency of micronucleated polychromatic erythrocytes (MPCE) were determined at approximately 24 and 48 hours after the dose administration. Two thousand PCE per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCE and normochromatic erythrocytes (NCE) in the first 200 erythrocytes for each animal. There were no statistically significant increases in the number of MPCE in the treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups. Reduction in the percentage of PCE was used as an indication of toxicity. No significant reduction (more than 20% versus that of the vehicle control) in the percentage of PCE was observed at any dose level or harvest time.

The frequency of MPCE in each treatment group was compared to that in the respective vehicle control using a one-tailed Student's T-test. The vehicle and positive control groups met the criteria for a valid test. The data from all dose groups treated with EDDN, at both bone marrow harvest time points, were not significant when compared to the vehicle control group. Therefore, the results of this assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, the test article was negative in the Micronucleus Assay. It was concluded that the test article did not cause chromosome damage *in vivo* and, therefore is not considered to be a clastogenic agent.

TABLE OF CONTENTS

INTRODUCTION

This study was conducted by Jian Song, Ph.D. and Karen S. K. Shore, B.A., at SITEK Research Laboratories from August 18, 2009 to September 10, 2009. The experimental procedures used to perform this study were essentially those of J.A. Heddle (10), W. Schmid (11), K.H. Mavournin, et al (12), M. Hayashi, et al (13) and R.R. Tice and M.D. Shelby (14).

The purpose of this study was to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes (MPCE) in mouse bone marrow cells (10, 11). Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (12-14).

MATERIALS

TEST ARTICLE

1. Name:	Ethylenediamine Dinitrate (EDDN)
2. CAS No.:	20829-66-7
3. Provided by:	USA RDECOM, AMSRD-MSF
4. Batch/Lot No.:	ABY08L031S010
5. Physical Description:	Clear Liquid (500 mg/mL)
6. Shipping Conditions:	Refrigerated (1-5°C)
7. Date Received:	08/05/09
8. Storage Conditions:	Refrigerated (1-5°C)
9. Purity:	99.5%
10. Expiration Date:	Not available

The Certificate of Analysis is not available but the Sponsor's data indicated the purity of the test article is 99.5%.

CONTROL SUBSTANCES

Positive Control

Cyclophosphamide (CP), which induces micronucleus formation, was used at 80 mg/kg body weight (8.0 mg/mL x 10 mL/kg) by oral gavage in this study. The information on the CP used in this study is provided below:

1. Source:	Sigma Chemical Company
2. CAS Registry No.:	6055-19-2
3. Lot No.:	075K1661
4. Purity:	99.7% by HPLC
5. Storage Conditions:	Refrigerated (1-5°C)
6. Expiration Date:	May 2, 2011

Sterile water (SITEK Lot No. 108) was used to dissolve the positive control. The expiration for this lot was January 30, 2010.

Vehicle Control

Sterile deionized distilled water (ddH_2O) was used as the vehicle control.

The information on the deionized distilled water (ddH_2O) used in this study is provided below:

1. Source:	SITEK Research Laboratories
2. CAS No.:	7732-18-5
3. Lot No.:	108
4. Storage Conditions:	Room Temperature
5. Expiration Date:	January 30, 2010

TEST ANIMALS

Approximately 42-day-old, male and female, CD-1 mice were obtained from Harlan Sprague Dawley, Inc. (Frederick, MD).

The animals were housed three or five animals per cage during the study. Hardwood chip bedding, free of injurious substances, was used. The animals received Purina Certified Rodent Diet (Code No. 5002C, Lot Nos. MAY 09 09 1C, Brentwood, MO) and fresh tap water *ad libitum*. Levels of contaminants present in the food and water were within acceptable levels. Bedding was changed at least twice per week.

The animals were quarantined for at least 7 days for the Micronucleus Assay prior to dose administration. The animals were observed each day and the observations, temperature and humidity of the animal room were recorded in the study notebook. Throughout the trials, the animal room was maintained at 21-24°C and 45-70% relative humidity. A 12-hour diurnal light cycle was employed.

A total of 42 mice (21 males, 21 females) were used for the Range Finding Test. A total of 90 mice (45 males, 45 females) were used for the Micronucleus Assay.

EXPERIMENTAL PROCEDURE

DOCUMENTATION

Detailed documentation of the procedures, results and methods used for the analysis of the results of this study were entered into the study notebook. The study notebook also includes the original protocol, study report copies and all relevant communications with the Sponsor.

TEST SYSTEM IDENTIFICATION

All of the animals to be dosed received an ear tag with a number unique to the particular study. All of the cages were assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director and the animal study proposal number (09-01-02) approved by SITEK's IACUC. The microslides were labeled with SITEK's study number, the last three digits of the animal number as a code number and the date of slide preparation.

SOLUBILITY TEST

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test was performed.

The test article was tested for its solubility/miscibility in sterile deionized distilled water. A nonviscous liquid will be tested for miscibility in weight per volume. The solubility/miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test would consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article was dissolved or until 1.5 mL of vehicle had been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in aliquots of 0.5 mL until 5.0 mL had been added. The volume of vehicle required for complete dissolution, and any additional observations, would be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle would be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test would be conducted. 1.5 mL of vehicle in 0.1 mL increments would be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle would be added in 0.5 mL increments until 5.0 mL had been added. The resulting solution would be thoroughly mixed

and observed for miscibility. The test article would be rated as either "not miscible," "partially miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations would be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article would be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data were available, the solubility/miscibility test would not be performed.

RANDOMIZATION OF TEST ANIMALS

Upon arrival, the animals were randomly assigned to clean, polycarbonate cages. All of the animals were ear tagged with a number unique to the study. In the Range Finding Test, the animals were assigned to experimental groups (three animals per sex), without regard to body weight, prior to dose administration.

In the Micronucleus Assay, the animals were weighed and placed into weight groups. Each weight group spanned 1 gram. Using a computer-generated random matrix, the animals then were assigned sequentially from the weight groups to randomized cages corresponding to the treatment groups (five animals per sex per group).

PREPARATION OF TEST ARTICLE DOSING SOLUTIONS

The stock solutions for the Range Finding Test and the Micronucleus Assay were prepared as specified in the dilution scheme which was kept in the study notebook. The specified amount of test article was weighed and the required volume of water was added to reach the highest stock concentration. The remaining concentrations were made by subsequent dilution.

The dosing solutions were prepared by SITEK study personnel just prior to treatment. In all treatments, the amount of vehicle administered to the animals was limited to a level which would have no significant toxic effect. The strength and stability of the test article and the test article dosing solutions under the experimental conditions was not determined by SITEK Research Laboratories.

TREATMENT PROCEDURE

The animals were treated with a single dose by oral gavage. The positive control (CP) was also given as a single dose by oral gavage. The total dose volume administered to the animals was 10 mL/kg body weight for each test article dose level and vehicle control. 10 mL/kg body weight was used for the positive control.

RANGE FINDING TEST

In the Range Finding Test, treatment groups of three mice per sex were treated at six test article dose levels of 10, 50, 100, 500, 1000 and 2000 mg/kg body weight and one group was treated with the vehicle control.

The animals were observed for 3 days following dose administration for treatment-related clinical signs and/or death. Body weights were checked on the day of dosing (Day 1) and at the end of the 3-day observation period (Day 4). At the end of the 3-day observation period, the animals were euthanized. The body weight data were entered into the computer to calculate the mean body weight and percent change in the mean body weight, using a MS Office Excel 2003 spreadsheet program. Clinical signs were entered into the computer in tabular form to demonstrate frequency. Doses for the Micronucleus Assay were selected based on the results of the Range Finding Test.

MICRONUCLEUS ASSAY

The Micronucleus Assay was performed at 500, 1000 and 2000 mg/kg body weight for the males and females based on the results of the Range Finding Test.

The animals were randomized and placed into treatment groups of five mice per sex. Two such treatment groups were designated for each of the test article dose levels and the vehicle control per harvest (a total of 80 mice). One group was designated for the positive control (10 mice). Sterile deionized distilled water and CP were used as the vehicle control and positive control, respectively. The dose volume was 10 mL/kg body weight for the test article, vehicle control and positive control.

The mice were euthanized by CO_2 asphyxiation approximately 24 or 48 hours after the dose administration. Five male and five female mice were euthanized from each test article dose level and the vehicle control at each harvest time. Five male and five female mice treated with the positive control were euthanized 24 hours after treatment.

After each animal was euthanized, the groin area was cleansed with 70% ethanol, and the femurs were exposed by cutting into the skin and muscle of the thighs. The femurs were separated just above the kneecaps, and the heads of both femurs were removed with scissors. The bone marrow from the femurs was flushed into a disposable culture tube, containing 1.0 mL of fetal bovine serum, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes were centrifuged at 800 rpm for 5 minutes. The supernatant was removed, leaving approximately 0.1 mL of serum above the cell pellet. The cells were resuspended by flicking the tube until a homogeneous suspension was observed.

A small drop of the cell suspension was placed on the unfrosted end of a clean microslide and spread along the length of the slide. The slides were allowed to air dry, then fixed in methanol for 15 minutes and allowed to air dry again. The slides were stained in Wright-Giemsa stain for 2-4 minutes, then rinsed in distilled water, allowed to air dry completely, and mounted in Cytoseal using #1cover glasses. The backs of the slides were cleaned with methanol.

The slides were scored "blind" in order to avoid bias on the part of the scorers. First, the number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) among 200 erythrocytes (PCE + NCE) per animal was determined. The number of micronucleated polychromatic erythrocytes (MPCE) then was determined for 2000 PCE per animal (2).

STATISTICAL ANALYSIS

The data from the score sheets were consolidated into summary sheets and entered into a computer, using an MS Office Excel 2003 spreadsheet program. The dose group means were calculated for the percentage of PCE as well as the frequency of MPCE. A significant reduction in percent PCE (more than 20% versus that of the vehicle control) was used as an indication of toxicity.

Data were analyzed separately for male and female animals. The frequency of MPCE in each dose group was compared to that in the respective vehicle control group using a one-tailed Student's t-test (15). An Excel 2003 statistical package was used to calculate p values for the t-test. The results were considered significant if the p value was ≤ 0.025. Statistical analysis was not performed on values that were lower than or equal to those of the respective vehicle control. The Cochran-Armitage Test (trend test) (16) was used to analyze trends if the Student's t-test showed a positive result. The trend test was considered significant if the p value was ≤ 0.05. For this study no trend test was needed.

CRITERIA FOR A VALID ASSAY

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed 10.

2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.

3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

EVALUATION OF TEST RESULTS

Positive Response

The test article was considered to have caused a positive response in this assay if:

1. The test article showed a positive dose-response trend and a statistically significant increase ($P \leq 0.025$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article caused a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may have been compared to historical vehicle control data. For this study the data from 48-hour harvest in female mice have been compared to historical vehicle control data (see Table 14).

2. In the event there was no positive dose-response trend, at least two consecutive test doses must have produced a statistically significant increase in the number of MPCE.

Negative Response

The test article was considered to have caused a negative response if none of the test doses showed a statistically significant increase in the number of MPCE when compared to the vehicle control.

Equivocal Response

The test article was considered to have caused an equivocal response if the test article induced a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend. In such a case, a repeat assay would have been performed only with the approval of the Sponsor.

ARCHIVES

The raw data, documentation, protocol, protocol amendment/deviation and a copy of the Final Report, along with an electronic file containing data tables and the Final Report of the study, will be maintained by SITEK Research Laboratories at Dr. Kirby's private residence until arrangements are made to transfer them to the Sponsor.

RESULTS

SOLUBILITY TEST

The solubility test was not performed because the test article, Ethylenediamine Dinitrate (EDDN, 99.5%), was provide as a clear colorless solution in sterile deionized distilled water at 500 mg/mL and the Sponsor has specified water as solvent.

RANGE FINDING TEST

The changes in the mean body weight for each dose group, which occurred by the end of the 3-day observation period following dosing, are presented in Table 1 (Appendix I). The Range Finding Test was performed at 10, 50, 100, 500, 1000 and 2000 mg/kg. No abnormal clinical signs were observed in any animal at any dose level during the three day observation period. At the end of the 3-day observation period, no reduction in the mean body weight was observed in any dose group of either sex.

Clinical signs were observed in the 3-day observation period. The data are presented in Tables 2-9 (Appendix I).

MICRONUCLEUS ASSAY

Based on the toxicity results from the Range Finding Test, the doses selected for the Micronucleus Assay were 500, 1000 and 2000 mg/kg body weight for the males and females.

The percentage of PCE and the MPCE frequency were determined from bone marrow preparations from animals sacrificed approximately 24 and 48 hours after dose administration. Individual results for the dose levels and vehicle groups in the Micronucleus Assay for the animals euthanized approximately 24 and 48 hours after treatment are presented for the males in Tables 10 and 12, respectively, and summarized in Table 14 and for the females in Tables 11 and 13, respectively, and summarized in Table 15 (Appendix I).

No significant reduction of the percentage of PCEs was observed at any test article dose level or harvest time in either sex. Animal C3650 in the male group dosed at 2000 mg/kg, died immediately prior to the 48 hour bone marrow harvest time. No wounds, swelling or other abnormal observations were made. The bone marrow was harvested and evaluated. The results were comparable to the other four animals in the group. Animal C3650 had 37.0% PCE and 2 MPCE per 2000 PCE, whereas the other four animals had a range of 31-38% PCE and 1-3 MPCE per 2000 PCE. Since no significant deviation from the remaining animals in the group was observed, the data from C3650 was included in this study.

The ranges of the mean numbers of MPCE in 2000 PCE from this assay are summarized below:

	Mean Numbers of MPCE in 2000 PCE in Males	
Treatment	24 Hours	48 Hours
Vehicle Control	0.6	2.4
Test Article Doses (500-2000 mg/kg body weight)	0.2-0.6	1.0-2.2
Positive Control-CP (80 mg/kg body weight)	50.2*	N/A

	Mean Numbers of MPCE in 2000 PCE in Females	
Treatment	24 Hours	48 Hours
Vehicle Control	0.8	2.2
Test Article Doses (500-2000 mg/kg body weight)	0.2-0.6	1.4-2.0
Positive Control-CP (80 mg/kg body weight)	48.6*	N/A

*Statistically significant response using a one-tailed Student's t-test

There was no evidence of a significant increase in the number of MPCE in the test article-treated groups at any dose level or harvest time as compared to the concurrent vehicle control groups. Mean numbers of MPCE for both males and females in the 48 hour treatment group was higher than SITEK's historical vehicle control data. This does not affect the result of the assay because the test article treated groups had fewer MPCE than the control.

CP, the positive control, showed a statistically significant increase in MPCE (Tables 10 and 11). The mean numbers of MPCE in 2000 PCE were 50.2 and 48.6 for the male and female mice, respectively, thus fulfilling the criteria for a valid assay.

All remaining criteria for a valid assay were also met. In the vehicle control, the average number of MPCE per 2000 PCE did not exceed 10. Five animals from each sex were alive at the time of euthanasia for each dose level, except for the male that died immediately prior to euthanasia in the 2000 mg/kg 48 hour harvest group. The data collected from the animal was not significantly different from the other four animals in the same group and was included in this study.

CONCLUSIONS

The test article, Ethylenediamine Dinitrate (EDDN, 99.5% pure), was tested for its potential to induce MPCE in the bone marrow cells of male and female CD-1 mice.

The results of the assay indicate that, under the conditions of this test and according to the criteria set for evaluating the test results, EDDN was negative at doses of 500, 1000 and 2000 mg/kg body weight for males and females in the Micronucleus Assay. Therefore, it was concluded that EDDN did not cause chromosome damage *in vivo* and is not considered to be a clastogenic agent. The strength and stability of the test article dosing solutions, under the experimental conditions, were not determined and the impact of the results on the conclusion is not known.

REFERENCES

1. United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

2. OECD Guideline for the Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

4. United States Food and Drug Administration, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

5. United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

6. Japanese Ministry of Agriculture, Forestry and Fisheries, 11 Nohsan, Notification No. 6283, October 1, 1999.

7. Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

8. Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

9. Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45 [ENV/MC/CHEM(98)17], Paris 1998.

10. Heddle, J. A. A rapid in vivo test for chromosomal damage. Mutat. Res., 18, 187-190, 1973.

11. Schmid, W. The micronucleus test. Mutat. Res., 31, 9-15, 1975.

12. Mavournin, K. H., et al. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res., 239, 29-80, 1990.

13. Hayashi, M., et al. In vivo rodent erythrocyte micronucleus assay. Mutat. Res., 312, 293-304, 1994.

14. Tice, R. R. and M. D. Shelby. Report of in vivo subgroup. Mutat. Res., 312, 287-292, 1994.

15. Lovell, D. P., et al. Statistical analysis of in vivo cytogenetic assays in: D. J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge. pp. 184-232, 1989.

16. Margolin, B. H., et al. Statistical analysis for the in vitro cytogenetic assay using Chinese hamster ovary cells. Enviro. Mutagen., 8, 183-204, 1986.

APPENDIX I

DATA TABLES

TABLE 1

RESULTS OF RANGE FINDING TEST

MOUSE BODY WEIGHTS INITIALLY AND APPROXIMATELY 72 HOURS FOLLOWING ORAL ADMINISTRATION OF ETHYLENEDIAMINE DINITRATE (EDDN)

STUDY NO.: 1003-1521 **VEHICLE: Water (10 mL/kg)**

MALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
	31	31	31	32	35	32	31
	31	31	33	34	33	30	33
	33	33	34	32	34	33	33
MEAN BODY WEIGHT	32	32	33	33	34	32	32

MALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
	34	32	33	34	36	33	27
	33	30	34	35	33	31	33
	32	34	33	33	35	35	29
MEAN BODY WEIGHT	33	32	33	34	35	33	30
% CHANGE MEAN BODY WEIGHT	3.1%	0.0%	0.0%	3.0%	2.9%	3.1%	-6.3%

FEMALE	BODY WEIGHTS AT 0 HOURS*						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
	28	30	29	27	29	26	28
	32	28	30	27	28	30	27
	26	26	30	26	25	27	26
MEAN BODY WEIGHT	29	28	30	27	27	28	27

FEMALE	BODY WEIGHTS AT 72 HOURS						
	DOSE LEVELS (mg/kg)						
	VEHICLE	10	50	100	500	1000	2000
	28	33	30	27	29	26	27
	32	28	31	27	29	31	28
	26	27	30	28	27	27	27
MEAN BODY WEIGHT	29	29	30	27	28	28	27
% CHANGE MEAN BODY WEIGHT	0.0%	3.6%	0.0%	0.0%	3.7%	0.0%	0.0%

NOTE: Body weights are in grams. Only surviving animals are used for calculating %Change Mean Body Weight.

* Body Weights were taken prior to dosing.

Reviewed by: QA PK SD 2/25/10

TABLE 2

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: MALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 1**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other -Cyanotic							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA RK SD 2/25/10

TABLE 3

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: FEMALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 1**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other-Cyanotic							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA RK SD 2/25/10

TABLE 4

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: MALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 2**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level. .

Reviewed by: QA RK SD 2/25/10

TABLE 5

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: FEMALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 2**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level..

Reviewed by: QA RK SD 2/25/10

TABLE 6

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: MALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 3**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA RK SD 2/25/10

TABLE 7

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: FEMALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 3**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA PK SD 2/25/10

TABLE 8

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: MALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 4**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA KK SD 2/25/10

TABLE 9

RESULTS OF RANGE FINDING TEST

CLINICAL SIGNS OBSERVED

SITEK Study No.: 1003-1521 **SEX: FEMALE**

Test Article: Ethylenediamine Dinitrate (EDDN) **DAY: 4**

SIGNS	DOSE LEVELS (mg/kg)**						
	Vehicle*	10	50	100	500	1000	2000
Unusual Appearance							
- Paralysis							
- Prostration							
- Ataxia							
- Piloerection							
- Closed Eyes							
- Hunched Back							
Unusual Body Secretions							
- Nasal Discharge							
- Lacrimation							
- Salivation							
- Bloody Stool							
- Diarrhea							
Abnormal Behavior							
- Convulsions							
- Inactivity							
- Tremors							
Breathing Difficulties							
- Labored Breathing							
Other							
No Signs Observed	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Death							

* Vehicle used for this study was water (10 mL/kg).
** 3 mice per sex dosed at each dose level.

Reviewed by: QA RK SD 2/25/10

TABLE 10

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE ORALLY ADMINISTERED
ETHYLENEDIAMINE DINITRATE (EDDN)
24 HOURS AFTER TREATMENT

STUDY NO.: 1003-1521 **Vehicle: WATER (10 mL/kg)**

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C3689	112	88	56.0	0	
VEHICLE	C3720	98	102	49.0	1	
VEHICLE	C3714	103	97	51.5	0	
VEHICLE	C3708	84	116	42.0	1	
VEHICLE	C3654	84	116	42.0	1	
	MEAN	96	104	48.1	0.6	
500	C3713	132	68	66.0	0	
500	C3704	88	112	44.0	0	
500	C3710	106	94	53.0	1	
500	C3674	109	91	54.5	0	
500	C3681	64	136	32.0	0	
	MEAN	100	100	49.9	0.2	*
1000	C3705	110	90	55.0	0	
1000	C3688	88	112	44.0	1	
1000	C3658	98	102	49.0	1	
1000	C3665	84	116	42.0	0	
1000	C3675	102	98	51.0	1	
	MEAN	96	104	48.2	0.6	*
2000	C3726	75	125	37.5	0	
2000	C3723	92	108	46.0	0	
2000	C3659	74	126	37.0	0	
2000	C3698	81	119	40.5	0	
2000	C3683	92	108	46.0	1	
	MEAN	83	117	41.4	0.2	*
CP**	C3731	79	121	39.5	39	
CP**	C3653	67	133	33.5	46	
CP**	C3732	69	131	34.5	45	
CP**	C3657	61	139	30.5	69	
CP**	C3724	82	118	41.0	52	
	MEAN	72	128	35.8	50.2	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by: QA RK SD 2/25/10

TABLE 11

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE ORALLY ADMINISTERED
ETHYLENEDIAMINE DINITRATE (EDDN)
24 HOURS AFTER TREATMENT

STUDY NO.: 1003-1521 Vehicle: WATER (10 mL/kg)

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE***
VEHICLE	C3716	114	86	57.0	0	
VEHICLE	C3669	89	111	44.5	1	
VEHICLE	C3661	107	93	53.5	1	
VEHICLE	C3673	128	72	64.0	1	
VEHICLE	C3656	82	118	41.0	1	
	MEAN	104	96	52.0	0.8	
500	C3715	96	104	48.0	0	
500	C3666	92	108	46.0	0	
500	C3696	94	106	47.0	1	
500	C3671	88	112	44.0	1	
500	C3690	88	112	44.0	0	
	MEAN	92	108	45.8	0.4	*
1000	C3722	102	98	51.0	0	
1000	C3733	88	112	44.0	0	
1000	C3652	106	94	53.0	0	
1000	C3718	100	100	50.0	0	
1000	C3706	116	84	58.0	1	
	MEAN	102	98	51.2	0.2	*
2000	C3702	96	104	48.0	1	
2000	C3678	95	105	47.5	0	
2000	C3703	82	118	41.0	0	
2000	C3668	98	102	49.0	1	
2000	C3663	84	116	42.0	1	
	MEAN	91	109	45.5	0.6	*
CP**	C3662	79	121	39.5	54	
CP**	C3676	88	112	44.0	53	
CP**	C3697	94	106	47.0	47	
CP**	C3691	62	138	31.0	43	
CP**	C3672	64	136	32.0	46	
	MEAN	77	123	38.7	48.6	<0.001

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

*** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by: QA RK SD 2/25/16

TABLE 12

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF MALE MICE ORALLY ADMINISTERED
ETHYLENEDIAMINE DINITRATE (EDDN)
48 HOURS AFTER TREATMENT

STUDY NO.: 1003-1521 **Vehicle: WATER (10 mL/kg)**

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE**
VEHICLE	C3682	92	108	46.0	2	
VEHICLE	C3684	64	136	32.0	2	
VEHICLE	C3664	71	129	35.5	2	
VEHICLE	C3727	92	108	46.0	4	
VEHICLE	C3701	99	101	49.5	2	
	MEAN	84	116	41.8	2.4	
500	C3685	96	104	48.0	0	
500	C3670	74	126	37.0	1	
500	C3707	97	103	48.5	1	
500	C3700	72	128	36.0	1	
500	C3699	80	120	40.0	2	
	MEAN	84	116	41.9	1.0	*
1000	C3647	78	122	39.0	1	
1000	C3728	80	120	40.0	1	
1000	C3717	97	103	48.5	1	
1000	C3655	84	116	42.0	2	
1000	C3711	80	120	40.0	1	
	MEAN	84	116	41.9	1.2	*
2000	C3730	73	127	36.5	3	
2000	C3650***	74	126	37.0	2	
2000	C3677	76	124	38.0	3	
2000	C3709	71	129	35.5	2	
2000	C3734	62	138	31.0	1	
	MEAN	71	129	35.6	2.2	*

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.
** The results are considered statistically significant if the p-value is less than or equal to 0.025.
*** Animal died immediately prior to bone marrow harvest. Results are comparable to the other four animals in this group.

Reviewed by: QA _____ SD 2/25/10

TABLE 13

MICRONUCLEUS ASSAY
PERCENT PCE AND INCIDENCE OF MPCEs
IN BONE MARROW OF FEMALE MICE ORALLY ADMINISTERED
ETHYLENEDIAMINE DINITRATE (EDDN)
48 HOURS AFTER TREATMENT

STUDY NO.: 1003-1521 **Vehicle: WATER (10 mL/kg)**

DOSE (mg/kg)	ANIMAL NUMBER	CELL COUNTS PCE	CELL COUNTS NCE	PERCENT PCE	MPCE per 2000 PCE	P VALUE FOR MPCE**
VEHICLE	C3712	95	105	47.5	2	
VEHICLE	C3692	96	104	48.0	0	
VEHICLE	C3729	78	122	39.0	3	
VEHICLE	C3719	94	106	47.0	3	
VEHICLE	C3725	83	117	41.5	3	
	MEAN	89	111	44.6	2.2	
500	C3679	92	108	46.0	1	
500	C3649	110	90	55.0	2	
500	C3686	98	102	49.0	0	
500	C3651	91	109	45.5	2	
500	C3680	99	101	49.5	2	
	MEAN	98	102	49.0	1.4	*
1000	C3667	84	116	42.0	1	
1000	C3693	106	94	53.0	1	
1000	C3695	90	110	45.0	1	
1000	C3645	96	104	48.0	2	
1000	C3646	82	118	41.0	2	
	MEAN	92	108	45.8	1.4	*
2000	C3721	78	122	39.0	4	
2000	C3694	98	102	49.0	1	
2000	C3648	81	119	40.5	3	
2000	C3660	96	104	48.0	2	
2000	C3687	82	118	41.0	0	
	MEAN	87	113	43.5	2.0	*

* Since mean value of treatment group was lower than or equal to vehicle control, the t-test was not done.

** The results are considered statistically significant if the p-value is less than or equal to 0.025.

Reviewed by: QA PK SD 2/25/10

TABLE 14

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Male Mice Orally Administered
Ethylenediamine Dinitrate (EDDN)

Study No.: 1003-1521 **Vehicle: Water (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	96	104	48.1	-	0.6
24	500	100	100	49.9	3.7 %	0.2
24	1000	96	104	48.2	0.2 %	0.6
24	2000	83	117	41.4	-13.9 %	0.2
24	CP*	72	128	35.8	-25.6 %	50.2 **
48	Vehicle	84	116	41.8	-	2.4
48	500	84	116	41.9	0.2 %	1.0
48	1000	84	116	41.9	0.2 %	1.2
48	2000	71	129	35.6	-14.8 %	2.2

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

Reviewed by: QA PK SD 2/25/10

TABLE 15

SUMMARY OF MICRONUCLEUS ASSAY RESULTS
Mean Percent PCE and Incidence of
MPCEs in Bone Marrow of Female Mice Orally Administered
Ethylenediamine Dinitrate (EDDN)

Study No.: 1003-1521 **Vehicle: Water (10 mL/kg)**

Time (hours)	Dose (mg/kg)	Cell Counts		PERCENT PCE	Change in %PCE***	MPCE for 2000 PCE
		PCE	NCE			
24	Vehicle	104	96	52.0	-	0.8
24	500	92	108	45.8	-11.9 %	0.4
24	1000	102	98	51.2	-1.5 %	0.2
24	2000	91	109	45.5	-12.5 %	0.6
24	CP*	77	123	38.7	-25.6 %	48.6 **
48	Vehicle	89	111	44.6	-	2.2
48	500	98	102	49.0	9.9 %	1.4
48	1000	92	108	45.8	2.7 %	1.4
48	2000	87	113	43.5	-2.5 %	2.0

NOTE: Five animals were used per group.

* CP was used as positive control and was dosed at 80 mg/kg by oral gavage.

** These results are considered statistically significant because the p-value is less than or equal to 0.025.

*** Change of Percent PCE in comparison with concurrent vehicle, calculated by the following formula:

$$\frac{\text{Percent PCE for Test Dose} - \text{Percent PCE for vehicle}}{\text{Percent PCE for vehicle}} \times 100$$

Reviewed by: QA RK SD 2/25/10

APPENDIX II

SITEK's HISTORICAL DATA FOR VEHICLE AND POSITIVE CONTROLS

MOUSE MICRONUCLEUS
HISTORICAL VEHICLE CONTROL DATA

Study Number	Vehicle	Date	Harvest Time	MPCE/ 2000 PCE Males	n=*	MPCE/ 2000 PCE Females	n=*
0849-1521	0.5% CMC	10/19/04	24 hrs.	0.0	1	0.0	1
			48 hrs.	0.2	2	0.2	2
0860-1521	Water	02/11/05	24 hrs.	0.8	3	0.8	3
			48 hrs.	0.8	4	0.4	4
0872-1521	UDCA	05/13/06	24 hrs.	0.4	5	0.6	5
			48 hrs.	0.6	6	0.4	6
0902-1521 B2	PBS	05/07/06	24 hrs.	0.6	7	1.0	7
0902-1521 B3	PBS	05/07/06	24 hrs.	0.6	8	0.8	8
0907-1521	Vehicle[B]	04/13/06	24 hrs.	0.4	9	0.8	9
0912-1521	0.5% CMC	08/08/06	24 hrs.	0.2	10	0.6	10
			48 hrs.	0.6	11	0.6	11
0917-1521	0.1%CMC, 0.9% Saline,	11/08/06	24 hrs.	0.6	12	0.8	12
	2% Tween80		48 hrs.	0.8	13	0.6	13
0922-1521		02/17/07	24 hrs.	0.0	14	0.6	14
0923-1521	0.9% Salline	02/15/07	24 hrs.	0.2	15	0.4	15
			48 hrs.	0.2	16	0.8	16
0926-1521	Corn Oil	02/22/07	24 hrs.	0.0	17	0.6	17
			48 hrs.	1.0	18	0.2	18
0954-1521	2% Polysorbate 80, 0.1% CMC,	07/24/07	24 hrs.	0.4	19	0.4	19
	0.9%NaCl		48 hrs.	0.0	20	0.4	20
0959-1521	Water	01/03/08	24 hrs.	0.0	21	0.2	21
			48 hrs.	0.4	22	0.2	22
0960-1521	Water	01/21/08	24 hrs.	0.6	23	0.2	23
			48 hrs.	0.2	24	0.4	24
0961-1521	Water	12/21/07	24 hrs.	0.2	25	0.6	25
			48 hrs.	0.6	26	0.2	26
0962-1521	Water	01/25/08	24 hrs.	0.2	27	0.2	27
			48 hrs.	0.8	28	0.6	28
0977-1521	Water	04/30/08	24 hrs.	0.6	29	0.0	29
			48 hrs.	0.8	30	0.0	30
0976-1521	Water	04/20/08	24 hrs.	0.6	31	0.6	31
			48 hrs.	0.4	32	0.0	32
0983-1521[A]	DMSO	06/19/08	24 hrs.	0.4	33		
			48 hrs.	0.4	34		
0957-1521	DMSO	05/07/08	24 hrs.	0.6	35	0.2	33
			48 hrs.	0.4	36	0.2	34
0958-1521	DMSO	05/25/08	24 hrs.	0.4	37	1.2	35
			48 hrs.	0.2	38	1.4	36
0984-1521	DMSO	09/09/08	24 hrs.	0.0	39	0.2	37
			48 hrs.	0.2	40	0.0	38

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.
[A] These studies included only one sex.
[B] 10% Povidone K30, 0.5% SLS and 0.05% Dow Corning® Antifoam 1510-US in purified water.

MEAN	0.5	n=*	40	0.5	n=*	38
S.D.	0.37			0.42		

MOUSE MICRONUCLEUS
HISTORICAL VEHICLE CONTROL DATA
MPCE per 2000 PCEs
October 2004 to July 2009

	MPCE/ 2000 PCE Males	MPCE/ 2000 PCE Females
Mean	0.5	0.5
Std. Dev.	0.37	0.42
Range	0 - 1.0	0 - 1.4
n=*	40	38

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

MOUSE MICRONUCLEUS
HISTORICAL POSITIVE CONTROL DATA

Study Number	Positive Control	Date	MPCE/ 2000 PCE Males	n=[B]	MPCE/ 2000 PCE Females	n=[B]
0849-1521	Cyclophosphamide	10/19/04	61.2	1	45.0	1
0860-1521	Cyclophosphamide	02/11/05	51.6	2	71.8	2
0872-1521	Cyclophosphamide	05/13/06	54.6	3	70.8	3
0902-1521 B2	Cyclophosphamide	05/07/06	36.6	4	47.8	4
0902-1521 B3	Cyclophosphamide	05/07/06	52.6	5	46.8	5
0907-1521	Cyclophosphamide	04/13/06	28.8	6	38.4	6
0912-1521	Cyclophosphamide	08/08/06	40.2	7	34.0	7
0917-1521	Cyclophosphamide	11/08/06	27.8	8	24.0	8
0922-1521	Cyclophosphamide	02/17/07	28.4	9	32.2	9
0923-1521	Cyclophosphamide	02/15/07	18.2	10	34.2	10
0926-1521	Cyclophosphamide	02/22/07	33.2	11	19.6	11
0954-1521	Cyclophosphamide	07/24/07	52.6	12	52.4	12
0959-1521	Cyclophosphamide	01/03/08	36.8	13	44	13
0960-1521	Cyclophosphamide	01/21/08	36.4	14	31	14
0961-1521	Cyclophosphamide	12/21/07	24.8	15	52	15
0962-1521	Cyclophosphamide	01/25/08	59.4	16	46.8	16
0976-1521	Cyclophosphamide	04/20/08	46.8	17	31.6	17
0977-1521	Cyclophosphamide	04/30/08	28.8	18	23.2	17
0983-1521[A]	Cyclophosphamide	06/19/08	37.2	19		
0957-1521	Cyclophosphamide	05/07/08	41.8	20	44.2	19
0958-1521	Cyclophosphamide	05/28/08	35.2	21	31.6	20
0984-1521	Cyclophosphamide	09/09/08	36.4	22	30.6	21
	Mean		39.5	n=[B] 22	40.6	n=[B] 21
	Std. Dev.		11.75		13.89	

[A] These studies included only one sex.

[B] "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

MOUSE MICRONUCLEUS
HISTORICAL POSITIVE CONTROL DATA
(CP 80 mg/kg)
MPCE per 2000 PCEs
October 2004 to July 2009

	MPCE/ 2000 PCE Males	MPCE/ 2000 PCE Females
Mean	39.5	40.6
Std. Dev.	11.75	13.89
n=*	22	21
Range	18.2 - 76.6	19.6 - 71.8

* "n" denotes the accumulated number of groups harvested for calculation of the mean and standard deviation.

APPENDIX III

STUDY PROTOCOL, PROTOCOL AMENDMENTS AND PROTOCOL DEVIATION

IN VIVO TEST FOR CHEMICAL INDUCTION OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES IN MOUSE BONE MARROW CELLS

This protocol is presented in two parts. Part One is designed to collect specific information pertaining to the test article and study. Part Two describes the study design in detail. **Please complete all bolded sections in Part One and sign Section 10 to approve the protocol.**

PART ONE

1.0 SPONSOR

1.1 Name: **USA RDECOM, AMSRD-MSF**

Environmental Acquisition & Logistics Sustaining Program

1.2 Address: **Aberdeen Proving Ground, MD 21010**

1.3 Sponsor's Study Coordinator: **Gunda Reddy, Ph.D., DABT**

2.0 TESTING FACILITY

2.1 Name: SITEK Research Laboratories

2.2 Address: 15235 Shady Grove Road, Suite 303
Rockville, Maryland 20850

2.3 Study Director: Jian Song, Ph.D.

3.0 STUDY NUMBERS

* 3.1 Testing Facility's Study No.: 1003-1521

3.2 Sponsor's Study No.: **Not Available**

4.0 TEST ARTICLE

GLP's require that test article characterization information must be provided in the final report. This includes identification, lot number, purity, stability, source, and expiration date. As per regulatory requirements, lack of the above information will be cited as a GLP violation in the "Study Director's Compliance Statement" section of the final report.

* To be completed by the Testing Facility.

4.1 Identification

Name: Ethylenediamine dinitrate (EDDN)

Batch/Lot No.: ABY08L031S010

4.2 Description

Color: Clear

Physical Form: Liquid (500 mg/mL in deionized, distilled water)

4.3 Analysis

Purity Information: 99.5%

Does the Sponsor require the use of a correction factor to account for impurity?

____ Yes **_X_ No**

If yes, what is the correction factor? _____

Determination of the test article characteristics as defined by Good Laboratory Practices will be the responsibility of the Sponsor. The specific GLP references for U.S. agencies are: FDA = 21 CFR, 58.105; EPA TSCA = 40 CFR, 792.105 and EPA FIFRA = 40 CFR 160.105.

4.4 Stability

Storage Conditions (check one):

__ Dry/Room Temperature **_X_ Refrigerated (1-5°C)**

__ Frozen (-10 to -20°C)

__ Other (please specify): _____

Expiration Date: Not Available

4.5 Preferred Vehicle (check one):

_X_ H_2O ____ Saline ____ Corn Oil ____ DMSO __ Ethanol (50% v:v in H_2O)

____ Other (please specify): _____

____ To be decided by the Testing Facility

4.6 Special Handling Instructions:

Use Standard Laboratory Safety Practices For Avoiding Exposure To Hazardous Substances And Follow Requirement For Explosive Material.

5.0 TEST ANIMALS AND TEST ARTICLE ADMINISTRATION

5.1 Test Animals (please check):

5.1.1 For the Range Finding Test.

____ Males ____ Females __X__ Both

5.1.2 For the Micronucleus Assay.

____ Males ____ Females __X__ Both

5.2 Route of Test Article Administration (check one):

_____ IP Injection __X__ Oral Gavage

The test article and vehicle will be administered by IP injection or oral gavage as indicated above, either directly or through a vehicle compatible with the test system. These routes of administration are valid methods for introduction of the test article. If necessary, other appropriate methods can be used at the request of the Sponsor.

_____ Other ** (please specify): _____

5.3 Frequency of Test Article and Vehicle Control Administration (check one):

__X__ Single administration and multiple harvests (approximately ____ 24, 48 and 72 hours or __X__ 24 and 48 hours after dose administration) (check one).

_____ Multiple administrations on ___ 2 or ___ 3 (check one) consecutive days and single harvest (approximately 24 hours after the last dose administration).

5.4 Volume of Administration

4.0 mL/kg will be administered when DMSO is used as the vehicle. 10-20 mL/kg will be administered when water, saline, corn oil, or other nontoxic materials are used as the vehicles.

** Additional charges will apply.

6.0 REGULATORY AGENCY SUBMISSION

6.1 Test Design Specifications

This study protocol is designed to meet or exceed the U.S. EPA, ICH and OECD Guidelines specified in the following documents (1, 2, 3):

United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

OECD Guideline for Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

6.2 Good Laboratory Practices

This study will be conducted in compliance with the following Good Laboratory Practice standards:

United States Environmental Protection Agency, Title 40 Code of Federal Regulations Parts 160 and 792, Revised July 1, 2005.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Revised April 1, 2005.

Japanese Ministry of Agriculture, Forestry and Fisheries, 11 NohSan, Notification No. 6283, October 1, 1999.

Japanese Ministry of Health and Welfare, Ordinance No. 21, April 1, 1997.

Japanese Ministry of International Trade and Industry, Notification No. 85, Basic Industries Bureau, March 31, 1984.

Organisation for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Environment Monograph No. 45[ENV/MC/CHEM (98)17], Paris 1998.

Will this study be submitted to a regulatory agency?

_X_ Yes **__ No**

If so, which agency(ies)? Worldwide

7.0 DOSING SOLUTIONS

The U.S. requirements for analysis of dosing solutions are specified in: FDA = 21 CFR, 58.113; EPA TSCA = 40 CFR, 792.113; EPA FIFRA = 40 CFR, 160.113, and OECD GLPs, Section 6.2.

Does the Sponsor want dosing solution analysis?

____ Yes** **_X_ No**

If yes, please complete the rest of this section.

If requested by the Sponsor, SITEK Research Laboratories will determine the strength and/or stability of the dosing solutions. Stability will only be determined if the dosing solutions are not prepared immediately prior to each use. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

Alternatively, the Sponsor will be responsible for determining the strength and/or stability of the dosing solutions.

Dosing solution analysis will be performed by:

____ SITEK Research Laboratories ____ Sponsor***

What dosing solutions will be analyzed?

From the Range Finding Test?

____ Yes ____ No

From the Assay?

____ Yes ____ No

Which concentration(s)? _____

** Additional charges will apply. See Special Services price schedule.

***Please note: All work pertaining to this study that is performed outside of SITEK is the responsibility of SITEK's Study Director. Therefore, as required by the GLPs, all of the following must be forwarded to the Study Director:**

- **All subcontract and/or Sponsor Quality Assurance audit findings and comments.**
- **Any deviations and/or amendments, if applicable.**
- **An original or copy of the analysis report.**
- **Location (address) of where the raw data from the analysis will be archived by the Sponsor or Subcontractor.**

If the subcontract work is not performed under the GLPs, a statement by the Sponsor informing SITEK's Study Director of such must be provided.

What amount of each concentration? _____

At what temperature should the dosing solutions be stored?

_____ Room Temperature _____ Frozen (-10 to -20 °C)

_____ Refrigerated (1-5 °C)

At what temperature should the dosing solutions be shipped?

_____ Room Temperature _____ On Wet Ice

_____ On Dry Ice

8.0 BLOOD (PLASMA) ANALYSIS FOR TEST ARTICLE CONCENTRATIONS

Does the Sponsor want blood (plasma) analysis?

_____ Yes **__X__ No**

Blood (plasma) analysis will be performed by:

_____ SITEK Research Laboratories _____ Sponsor

What blood (plasma) analysis will be performed?

From the Range Finding Test?

_____ Yes _____ No

From the Assay?

_____ Yes _____ No

Test Animals: Males only.

Time points (bleeding time after the dose administration), dose levels and number of animals for the blood sample collection (unless otherwise specified by the Sponsor).

From the Range Finding Test (if sufficient pharmacokinetic information is not available), blood samples will be taken after dose administration at 3 time points (to be determined by the Sponsor), at 3 dose levels starting from the highest dose, then every other dose level. One male per dose per time point and a total of 9 males will be used.

** Additional charges will apply. See Special Services price schedule.

From the Micronucleus Assay blood samples will be taken from all three dose levels. Only 1 time point will be used based on the plasma analysis results from the Range Finding Test or on the pharmacokinetic information of the test article (to be determined by the Sponsor). Three males per dose per time point and a total of 9 males will be used.

If requested by the Sponsor, SITEK Research Laboratories will determine the test article concentration in the plasma. The method of analysis may be provided by the Sponsor, or if requested by the Sponsor, SITEK Research Laboratories will develop the method of analysis.

9.0 STUDY DATES

* 9.1 Proposed Experimental Start Date: August 18, 2009

Defined as the date the animals are dosed for the Range Finding Test or Micronucleus Assay.

* 9.2 Proposed Experimental Completion Date: September 25, 2009

Defined as the last date on which data are collected directly from the study.

* 9.3 Proposed Draft Report Date: October 9, 2009

9.4 Final Report: The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter.

10.0 PROTOCOL APPROVAL

* _____ 8-5-2009
Study Director — Date

_____ August 5, 09
Sponsor's Study Coordinator — **Date**

* _____ 8-5-09
Quality Assurance Manager — Date

* _____ 8-5-09
Safety Officer — Date

* To be completed by the Testing Facility.

STUDY DESIGN

PART TWO

11.0 PURPOSE

The purpose of this study is to evaluate the test article for its potential to cause genetic damage as manifested by induced micronucleated polychromatic erythrocytes in mouse bone marrow cells (4,5).

12.0 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

Mice have been used extensively in the Micronucleus Assay and have been demonstrated to be effective in detecting the clastogenic activity of chemicals from a wide range of chemical classes (6, 7, 8).

13.0 ABBREVIATIONS

CP	-	Cyclophosphamide
DMSO	-	Dimethyl Sulfoxide
FBS	-	Fetal Bovine Serum
IACUC	-	Institutional Animal Care and Use Committee
IP	-	Intraperitoneal
MPCE	-	Micronucleated Polychromatic Erythrocytes
MTD	-	Maximum Tolerated Dose
NCE	-	Normochromatic Erythrocytes
PCE	-	Polychromatic Erythrocytes

14.0 ANIMALS

14.1 Protocol Approval

This protocol has been approved for the necessity of using laboratory animals by SITEK's Institutional Animal Care and Use Committee (IACUC) on February 24, 2006 (No. 06-02). In this regard, the number of animals used, the objectives of the study, the housing procedures, the rationale for animal use, the procedures involving animals, the potential for distress or pain for the animals, and the methods of euthanasia have been reviewed and approved.

14.2 Source

Forty-two-day-old (approximately), male and female CD-1 mice will be obtained from Harlan Sprague Dawley Inc. or other acceptable vendor which routinely monitor the animals for bacterial, viral and other murine infections. The animal body weight range will be approximately 16-38 grams on delivery.

14.3 Housing and Quarantine

When the animals arrive, they will be housed according to sex in clean cages of sufficient size to allow free movement. They will be placed, one sex at a time, up to ten animals per cage, into polycarbonate cages. Hardwood chip bedding free of injurious substances will be used. The animals will receive Purina Certified Rodent Diet and fresh tap water ad libitum. The levels of contaminants present in the food and water are very low and well within acceptable levels, and are not expected to affect the outcome of the study. Bedding will be changed at least twice a week.

The animals will be quarantined for at least 7 days prior to dose administration. The animals will be observed each day, and all observations and the temperature and humidity of the animal room will be recorded in the study notebook. The animal room will be maintained at 18-26 °C and 30-70% humidity. Occasionally, minor deviations may occur due to change of outside weather conditions. A 12-hour diurnal light cycle will be employed. Only animals that are certified as healthy will be used in the study.

15.0 TEST SYSTEM IDENTIFICATION

All animals to be treated will receive an ear tag with a number unique to the particular study. All cages will be assigned a cage card labeled in indelible ink with the following information: animal room number, animal receipt date, source, species/strain, sex, weight/age, number of animals per cage, SITEK's study number, Study Director, and the animal study proposal number approved by SITEK's IACUC. The microslides will be labeled with the study number, the last one, two, three or four digits of animal number as code number and date of slide preparation.

16.0 CONTROL SUBSTANCES

16.1 Positive Controls

Cyclophosphamide (CP), at 80 mg/kg (8.0 mg/mL X 10 mL/kg) as a single dose by oral gavage, will be dissolved in water and used as the positive control. The source and storage conditions for CP are given below:

Source: Sigma Chemical Company or other vendor Storage Conditions: 1-5°C CAS No.: 6055-19-2

The specific source, lot number and expiration date of the CP will be documented in the report.

If necessary, other appropriate positive controls can be used with the approval of the Sponsor.

16.2 Vehicle Controls

The vehicle used for dissolving or suspending the test article will be used as the vehicle control. Deionized, distilled water, saline, corn oil (CAS #80001-30-7), dimethyl sulfoxide (CAS #67-68-5), methyl cellulose (CAS 9004-67-5), hydroxypropylmethyl cellulose (CAS #9004-65-3), tween 80 (CAS #9005-65-6) and ethanol (CAS #64-17-5) are some of the vehicles, which are compatible with this test system. If these vehicles are not suitable, others may be used with the approval of the Sponsor. The source, lot number and storage conditions of the vehicle control will be documented in the report.

17.0 DOCUMENTATION

Detailed documentation of the procedures, results, and methods used for the analysis of the results of this study will be entered in a study notebook. The study notebook will also include the protocol, protocol amendments and deviations, copy of the study report, and all relevant communications with the Sponsor.

18.0 EXPERIMENTAL PROCEDURE

18.1 Determination of Solubility/Miscibility

In order to determine the appropriate vehicle for delivering the test article to the test system, or to determine the maximum achievable concentration in the vehicle requested by the Sponsor, a solubility/miscibility test will be performed.

The test article will be tested for its solubility/miscibility in deionized, distilled water, saline, DMSO, corn oil, ethanol and/or other appropriate vehicles. Solid and viscous liquid test articles will be tested for solubility in weight per volume, and nonviscous liquids will be tested for miscibility in volume per volume or weight per volume. The solubility/ miscibility test will be performed as described below.

For solid and viscous liquid test articles, the solubility test will consist of weighing out 25-100 mg aliquots of test article and adding vehicle in 0.1 mL increments, with thorough mixing between additions, until the test article is dissolved or until 1.5 mL of vehicle has been added to the vessel. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in aliquots of 0.5 mL until 5.0 mL has been added. The volume of vehicle required for complete dissolution, and any additional observations, will be recorded in the study workbook. Test articles that do not dissolve in 5.0 mL of vehicle will be recorded as either "not soluble," "partially soluble forming a homogeneous suspension," or "partially soluble not forming a homogeneous suspension."

For nonviscous liquid test articles, a miscibility test will be conducted. 1.5 mL of vehicle in 0.1 mL increments will be added to 0.5 mL aliquots of the test article. If the test article does not dissolve in 1.5 mL of vehicle, more vehicle will be added in 0.5 mL increments until 5.0 mL has been added. The resulting solution will be thoroughly mixed and observed for miscibility. The test article will be rated as either "not miscible," "partially

miscible," or "completely miscible" in each of the four preferred vehicles. The miscibility rating and any additional observations will be recorded in the study workbook.

Where solubility/miscibility cannot be achieved, the test article will be delivered as a suspension in the desired vehicle. If sufficient solubility/miscibility data are available, the solubility/miscibility test will not be performed.

18.2 Randomization

The animals will be randomized one sex at a time and placed into experimental groups as follows: In the Range Finding Test, the animals will be randomly assigned to experimental groups, without regard to body weight, prior to dose administration (three animals per group). In the Micronucleus Assay, the animals will be weighed and placed in weight groups of 1-gram differences. Using a computer generated random number matrix, the animals will be assigned sequentially, from low to high weight groups, to cages corresponding to treatment groups (five animals per group).

18.3 Preparation of Test Article

The stock solutions for the Range Finding Test and the Micronucleus Assay will be prepared as specified in the dilution scheme which will be kept in the study notebook. The highest stock solution will be prepared by mixing together the required weight/volume of the test article and the appropriate amount of the vehicle until complete solubilization or a homogeneous suspension has been achieved. The remaining doses specified in the dilution scheme will be prepared as in the case of the highest stock solution, by performing a subsequent dilution or by varying the volume administered from the highest stock concentration to the animals. When preparing the top dosing stock and any subsequent dosing stock with a viscous or non-viscous liquid, the test article should never be diluted more than 10-fold. The dosing solutions will be prepared by SITEK study personnel just prior to treatment and kept at room temperature. In all treatments, the amount of vehicle administered to the animals will be limited to a level which has no significant toxic effect. If necessary, the test article may be administered at full strength.

18.4 Range Finding Test

The dose levels for the Micronucleus Assay will be selected according to the toxicity of the test article and in consultation with the Sponsor. If sufficient information is not available on the toxicity of the test article, a Range Finding Test will be performed. The actual dose levels for the assay, once determined, will be added to the protocol in the form of an amendment. Unless otherwise specified by the Sponsor, the doses for the Range Finding Test are 2000, 1000, 500, 100, 50 and 10 mg/kg for solid and viscous test articles and 2.0, 1.0, 0.5, 0.1, 0.05 and 0.01 mL/kg for liquid test articles.

Treatment groups of three animals per sex will be dosed with the test article dosing solutions using the route, frequency and volume of administration selected in Section 5.0. One treatment group will be treated with only the vehicle. Food and water will be provided ad libitum. The animals will be carefully observed for 3 days after the single or the first administration of the test article. Daily records of all clinical observations and number of deaths (if any) will be kept for the treatment groups. Body weights will be checked on the day of dosing (Day 1) and by the end of the 3-day observation period (Day 4). All animals

will be euthanized at the end of the observation period. The body weights will be entered into a computer, using MS Excel 2003 Spreadsheet program. The clinical signs and mortality will be entered into a computer, using MS Word 2003.

In addition to the animals treated with the test article for the standard Range Finding Test, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Range Finding Test.

The animals will be euthanized by CO_2 asphyxiation. Blood will be taken by cardiac puncture.

Approximately 0.4-1.0 mL samples of blood will be drawn into a syringe containing heparin (10,000 units). The needles will be removed from the syringes, and the blood will be transferred to an appropriately labeled 1.5 mL microfuge tubes. The tubes will be capped and placed on wet ice. Labeling will include Study number, test article identification, dose level, animal number and sampling time.

Within 0.5 hour of collection, the samples will be centrifuged at a speed of 14,000 rpm for 2 minutes. Being careful not to transfer any of the red blood cell fractions, the plasma fractions will be transferred by pipet into 1.5 mL storage vials equipped with O-ring screw caps. The samples will be stored immediately at -10 to -20°C. The frozen plasma samples will be shipped overnight on dry ice.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma in order to pick the time point for the Micronucleus Assay.

The toxicity of the test article will be evaluated on the basis of a combination of factors, namely, number of deaths, loss of body weight, and other clinical symptoms observed during the 3-day period. If possible, the highest dose selected for the Micronucleus Assay will be the Maximum Tolerated Dose (MTD) at which no deaths are recorded but animals show evidence of toxicity and/or more than 10% loss of body weight. If no toxicity is observed, the maximum dose treated will be 2000 mg/kg or 2.0 mL/kg. In addition, two lower doses, preferably one-half and one-fourth of the high dose, will be included in the assay.

18.5 Micronucleus Assay

The animals will be randomized and placed into treatment groups of five males and/or five females. One or two treatment groups will be assigned to each dose level and vehicle control. The dose levels will be as determined by the Range Finding Test after consultation with the Sponsor. One treatment group will be assigned to the positive control.

Food and water will be provided ad libitum. The test article will be administered as indicated in Section 5.0. Appropriate vehicle and positive controls will be maintained.

The animals will be euthanized by CO_2 asphyxiation. One treatment group from each test article dose level and vehicle control will be euthanized approximately 24 and 48 hours or 24, 48 and 72 hours after dose administration. Only one euthanasia, 24 hours after the dose administration, will be made of the animals dosed with the positive control. If

multiple administrations and single harvest are selected, the animals will be euthanized approximately 24 hours after the last dose administration.

The number of animals to be used in the standard Micronucleus Assay will be as follows (if both sexes are used):

If single administration and multiple harvests are used:

For three harvests:

	No. of Animals Per Harvest Time						
Dose Level	24 Hours		48 Hours		72 Hours		
	Males	Females	Males	Females	Males	Females	Total
High	5	5	5	5	5	5	30
Mid	5	5	5	5	5	5	30
Low	5	5	5	5	5	5	30
Vehicle Control	5	5	5	5	5	5	30
Positive Control	5	5	N/A	N/A	N/A	N/A	10
							130

For two harvests:

	No. of Animals Per Harvest Time				
Dose Level	24 Hours		48 Hours		
	Males	Females	Males	Females	Total
High	5	5	5	5	20
Mid	5	5	5	5	20
Low	5	5	5	5	20
Vehicle Control	5	5	5	5	20
Positive Control	5	5	N/A	N/A	10
					90

If multiple administrations and single harvest are used:

	No. of Animals Per Group		
Dose Level	Males	Females	Total
High	5	5	10
Mid	5	5	10
Low	5	5	10
Vehicle Control	5	5	10
Positive Control	5	5	10
			50

After the animal has been euthanized, the groin area will be cleansed with 70% ethanol, and both femurs will be exposed by cutting into the skin and muscle of the thighs. The femurs will be separated just above the kneecaps, and the femur heads will be removed

with scissors. The bone marrow from both femurs will be flushed into a medium disposable culture tube (13x100 mm), containing 1.0 mL of FBS, using a 1-cc syringe fitted with a 25-gauge, 1" needle.

The tubes will be centrifuged at 800 rpm for 5 minutes, and the supernatant will be removed, leaving approximately 0.1 mL of serum above the cell pellet. The cell pellet will be resuspended in the remaining serum until a homogeneous suspension is observed.

A small drop of the cell suspension will be placed on the unfrosted end of a clean microslide and spread along the length of the slide. The slides will be allowed to air dry, fixed in methanol for 15 minutes, and allowed to air dry again. The slides will be stained in Wright-Giemsa stain for 2-4 minutes, rinsed in distilled water, allowed to air dry completely, and mounted in Cytoseal using #1 cover glasses. The backs of the slides will be cleaned with methanol.

The slides will be scored "blind" in order to avoid bias on the part of the scorer(s). The number of PCE among total erythrocytes (PCE + NCE) will be determined for each animal by counting a total of at least 200 erythrocytes. The number of MPCE then will be scored for 2000 PCE per animal (2).

In addition to the animals treated with the test article for the standard Micronucleus Assay, additional animals will be dosed for bleeding as indicated in Section 8.0, if blood samplings are needed from the Micronucleus Assay.

The animals will be euthanized by CO_2 asphyxiation. Blood will be taken by cardiac puncture.

The procedures for blood collection, storage and shipping will be the same as in the Range Finding Test.

The collection of blood samples from mice treated with several concentrations of the test article is necessary to determine the test article concentration in plasma. There is sufficient correlation for measurements of test article in plasma to be adequate for validating bone marrow exposure (3).

18.6 Statistical Analysis

The data will be analyzed separately for male and female animals. The data from the score sheets will be consolidated into summary sheets and entered into a computer using a MS Excel 2003 validated spreadsheet program. The treatment group means will be calculated for the percentage of PCE among total erythrocytes, as well as the frequency of MPCE. A significant reduction (more than 20% versus that of the control vehicle) will be used as an indication of toxicity. Unless otherwise indicated, the frequency of MPCE in each treatment group will be compared to that in the respective vehicle control using a one-tailed Student's t-test (9). MS Excel 2003 will be used to calculate p values for the t-test. The results will be considered significant if the p value is ≤ 0.025. The Cochran-Armitage Test (trend test) (10) will be used for evidence of a dose-related response, if the Student's t-test shows a positive result. The trend test will be considered significant if the p value is ≤ 0.05.

Historical vehicle control values also will be taken into consideration when necessary as described in Section 18.8.1. Statistical analysis will not be performed if the test dose value is equal to or less than the concurrent or historical vehicle control.

18.7 Criteria for a Valid Assay

1. In the vehicle control, the average number of MPCE per 2000 PCE should not exceed ten.

2. In the positive control, the increase in the average number of MPCE per 2000 PCE over the average number of MPCE for the vehicle control should be statistically significant.

3. At least five animals from each sex must be alive at the time of euthanasia for each dose level.

18.8 Evaluation of Test Results

18.8.1 Positive Response

The test article will be considered to have caused a positive response in this assay if:

1. The test article shows a positive dose-response trend and a statistically significant increase ($p \leq 0.025$) in the number of MPCE at one or more dose levels over that of the concurrent vehicle control. In the event that the test article causes a statistically significant increase in the number of MPCE due to an unusually low number of MPCE (less than 0.05%) in the concurrent vehicle control, the data from that dose may be compared to historical vehicle control data.

2. In the event there is no positive dose-response trend, at least two consecutive test doses show a statistically significant increase in the number of MPCE.

18.8.2 Negative Response

The test article will be considered to have caused a negative response if none of the test doses show a statistically significant increase in the number of MPCE when compared to the vehicle control.

18.8.3 Equivocal Response

If the test article induces a statistically significant increase in the number of MPCE when compared to the vehicle control at one of the test doses without a positive dose-response trend, the results will be considered equivocal. In such a case, a repeat assay will be performed with the approval of the Sponsor.

18.8.4 Other Considerations

The above criteria will be used as guidelines in evaluating the test results. However, the Study Director may take other factors into consideration in evaluating the test results.

19.0 PROTOCOL AMENDMENTS AND DEVIATIONS

If changes in the approved protocol are necessary, such changes will be documented in the form of protocol amendments and protocol deviations. Protocol amendments will be generated when changes in the protocol are made prior to performing a study or part of a study affected by the changes. In such cases, a verbal agreement to make such changes will be made between the Study Director and the Sponsor, and these changes and the reasons for them will be documented. Protocol deviations will be generated when the procedures used to perform the study do not conform to the approved protocol. The Sponsor will be informed of these deviations, and these changes, along with the reasons for them or explanations, will be documented. If the amendments or deviations involve animal use, they will be reviewed by the IACUC. Protocol amendments and deviations will be appended to the protocol.

20.0 REPORT OF RESULTS

20.1 Content

The results of the study will be submitted to the Sponsor in the form of a final report. A draft report will be submitted before the final report is issued. The final report will be initiated sixty days after remittance of the draft report and issued no later than thirty days thereafter. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study, and the dates on which the study was initiated and completed.

2. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

3. Statistical methods employed for analyzing the data.

4. The test and control articles identified by name, chemical abstracts number or code number, strength, purity and composition, or other appropriate characteristics.

5. A description of the methods used.

6. The name, sex and source of the animals used.

7. A description of the treatment procedures, vehicle used for treatment and duration of treatment.

8. A description of all circumstances that may have affected the quality or integrity of the data.

9. The name of the Study Director and the names of other technical personnel or other professionals, who participated in performing the study.

10. A description of the transformations, calculations or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

11. The signed and dated final report of the Study Director and Quality Assurance Manager.

12. The location where the raw data and final report are to be stored.

13. A statement from the Quality Assurance Unit.

20.2 Changes and Corrections to the Final Report

All changes to the final report will be in the form of report amendments and will include the reasons for the changes. Report amendments will be added to the final report as an addendum.

21.0 ARCHIVES

The raw data, documentation, protocol and Final Report, along with an electronic file containing the data tables and copy of the Final Report of the study will be maintained in SITEK Research Laboratories' Archives, 15235 Shady Grove Road, Suite 303, Rockville, Maryland, for ten years. All raw data, documentation, and the final report of all Subcontractors/Sponsor work will be maintained by the Subcontractor/Sponsor.

22.0 REFERENCES

1. United States Environmental Protection Agency, Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5395, *In Vivo* mammalian bone marrow cytogenetics tests: Micronucleus Assay. Revised July 1, 2002.

2. OECD Guideline for the Testing of Chemicals, No. 474. Mammalian Erythrocyte Micronucleus Test. Adopted July 21, 1997.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. Federal Register 61 (80):18198-18202, 1996.

4. Heddle, J. A. A rapid in vivo test for chromosomal damage. Mutat. Res., 18, 187-190, 1973.

5. Schmid, W. The micronucleus test. Mutat. Res., 31, 9-15, 1975.

6. Mavournin, K. H., et al. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res., 239, 29-80, 1990.

7. Hayashi, M., et al. In vivo rodent erythrocyte micronucleus assay. Mutat. Res., 312, 293-304, 1994.

8. Tice, R. R. and M. D. Shelby. Report of in vivo subgroup. Mutat. Res., 312, 287-292, 1994.

9. Lovell, D. P., et al. Statistical analysis of in vivo cytogenetic assays in: D. J. Kirkland (Ed.) Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III. Cambridge University Press, Cambridge. pp. 184-232, 1989.

10. Margolin, B. H., et al. Statistical analysis for the in vitro cytogenetic assay using Chinese hamster ovary cells. Enviro. Mutagen., 8, 183-204, 1986.

PROTOCOL AMENDMENT

Amendment No.:	1
Sponsor:	USA RDECOM, AMSRD-MSF Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	1003-1521
Sponsor's Study No.:	N/A
Test Article I.D.:	Ethylenediamine Dinitrate (EDDN)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Amendment No. 1: Protocol Page 11, Section 18.4 Range Finding Test. Based on the results of the Range Finding Test, the dose levels for the Micronucleus Assay will be 500, 1000 and 2000 mg/kg.

Reason for Amendment No. 1: The dose levels for the Micronucleus Assay were added to the protocol by amendment as stated in the protocol.

APPROVED:

Jian Song, Ph.D.
Study Director

8-25-09
Date

PROTOCOL AMENDMENT

Amendment No.:	2
Sponsor:	USA RDECOM, AMSRD-MSF Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	1003-1521
Sponsor's Study No.:	N/A
Test Article ID:	Ethylenediamine dinitrate (EDDN)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Amendment No. 2: Protocol Page 1, Section 2.3, Study Director, Jian Song, Ph.D. has been replaced by Paul E. Kirby, Ph.D. as Study Director.

Reason for Amendment No. 2: Jian Song, Ph.D. is no longer in the employ of SITEK Research Laboratories.

APPROVED:

Paul E. Kirby, Ph.D.
Study Director

2-25-10
Date

PROTOCOL DEVIATION

Deviation No.:	1
Sponsor:	USA-RDECOM, AMSRD-MSF Environmental Acquisition & Logistics Sustaining Program Aberdeen Proving Ground, MD 21010
Testing Facility:	SITEK Research Laboratories 15235 Shady Grove Road, Suite 303 Rockville, Maryland 20850
SITEK's Study No.:	1003-1521
Sponsor's Study No.:	N/A
Test Article I.D.:	Ethylenediamine Dinitrate (EDDN)
Protocol Title:	*In Vivo* Test for Chemical Induction of Micronucleated Polychromatic Erythrocytes in Mouse Bone Marrow Cells

Deviation No. 1: Protocol Page 15, Section 18.7 Criteria for a Valid Assay. One male in the 48 hour harvest group at 2000 mg/kg, died immediately prior to euthanasia. Since this animal had died at harvest time, it was decided to harvest and evaluate the bone marrow. There were no significant differences in the results in this animal and the other four animals in the group. The results were included in the data for this study.

Reason for Deviation No. 1: At least five animals from each sex must be alive at the time of euthanasia for each does level as stated in the protocol.

APPROVED:

Jian Song, Ph.D.
Study Director

8-27-09
Date